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HORMONAL MODULATION OF PHEROMONE-MEDIATED BEHAVIOR IN A CRUSTACEAN

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Abstract

A stereotyped courtship display is normally triggered in the male blue crab, *Callinectes sapidus,* by a pheromone released from pubertal females. Following bilateral eyestalk ligation, ablation, or optic tract transection, males do not respond to the pheromone, suggesting that neural pathways in the eyestalk ganglia are important for processing or transmitting pheromone stimulus information. Interestingly, males begin to exhibit spontaneous display behavior within a few days following eyestalk ligation or ablation, but not if only the optic tracts are transected. We propose that the loss of a circulating eyestalk factor, which moderates the activity of CNS pathways controlling courtship display, is responsible for the induction of the spontaneous behavior. This factor may normally control pheromone receptivity in males by modulating the excitability of these CNS pathways either directly or by acting via an intermediate(s); possibly by regulating the activity of the androgenic glands which exhibit massive hypertrophy following eyestalk ligation.

Introduction

A fundamental goal of neuroethology is to understand the mechanisms which regulate the "behavioral state" of an animal (Truman and Weeks, 1985). Such regulation assures that the organism's behavior represents an appropriate interface between its internal physiological condition and the external environment. Hormonal controls are frequently of major importance in this regulation, particularly in reproductive behavior; although depending on the animal's reproductive strategy, social and environmental cues also can be of paramount importance (Crews and Moore, 1986). Pheromone communication plays a critical role in the reproductive activities of animals in nearly all major phyla (Shorey, 1976). Frequently these chemical messages trigger very specific stereotyped behaviors, which ultimately insure that mating is successful. In this report we consider a mechanism by which internal factors may regulate sensory activation of such a behavioral program.

The reproductive behavior of *C. sapidus* is coordinated in part by a pheromone that is present in the urine of pubertal females and detected by males via chemoreceptor sensilla (aesthetascs) on the outer flagellum of the first antennae (Gleeson, 1980, 1982). In receptive males this pheromone triggers a stereotyped courtship display which is characterized by lateral spreading of the chelae, extension of the walking legs, and lateral waving of the swimming appendages above the carapace. Immediately following the display, the male grasps the female and carries her beneath him for up to several days until she undergoes her maturity molt. Copulation is initiated soon

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thereafter, and this is normally the only time during her entire life that the female will mate.

In experiments which have focused on determining the chemical nature of this pheromone, males are routinely screened for pheromone receptivity before use in bioassays (Gleeson *et al.,* 1984). Interestingly, this screening procedure has revealed apparent cycles of receptivity within the male population; *i.e.,* at times very few males will display when presented with a pheromone stimulus, whereas at other periods nearly all males are responsive. No obvious correlations between these peaks of receptivity and seasonal mating activity, molt stage, or cycles of environmental parameters have yet been identified.

This apparent periodicity in receptivity suggested that hormonal controls may be operating. If so, the androgenic glands might be potential sources of such a regulatory factor since they are known to directly control the development and maintenance of both primary and secondary sexual characteristics in male crustaceans (Charniaux-Cotton and Payen, 1985). Because an eyestalk hormone (possibly gonad inhibiting hormone) has been shown to moderate the activity of the androgenic glands in several crustacean species (Adiyodi and Adiyodi, 1970; Adiyodi, 1985), we reasoned that ligating the eyestalks in male blue crabs may be an approach to increasing androgenic hormone production and consequently enhancing the males' pheromone receptivity; *i.e.,* if, in fact, an androgenic hormone affects receptivity. This hypothesis is only testable, however, if the olfactory pathways in the eyestalk ganglia [*e.g.,* the medulla terminalis (Ache and Fuzessery, 1979)] are not critical for processing pheromone stimulus information.

In this study we present evidence suggesting the existence of hormonal modulation of pheromone-triggered courtship display behavior in *C. sapidus* and begin to define the neural substrate affected by this modulation.

Materials and Methods

All animals were obtained from commercial sources, maintained in holding tanks with flow-through seawater systems, and sustained on a mixed diet of fish, squid, and shrimp which was offered 2–3 times weekly. Molt stages were assessed according to the criteria of Van Engel (1958).

Eyestalk ligations were performed by tightly tying off each eyestalk at its base in the region of the arthrodial membrane using a suitable length of 000 suturing silk. In animals in which the eyestalks were ablated, amputations were made just distal to a ligature tied at the base of each eyestalk; this procedure considerably reduced the loss of hemolymph from the stump. The ligation and ablation procedures are equivalent in their effects in that with ligation there is an immediate loss of visual input as judged by the animal's lack of response to visual cues that would normally trigger a defense posture. Furthermore, necrosis of eyestalk tissue is quite apparent within 24 h post-ligation with the eyestalk frequently becoming detached after a few days. Transections of the optic tracts were achieved using small scissors (Mini-Vannas) which were inserted through a transverse slit, approximately 2 mm in length, made in the mesial surface of the arthrodial membrane at the base of each eyestalk.

For behavioral observations, all animals were held individually in 40-liter aquaria, each having a flow-through seawater supply. Behavioral activity was monitored in one of two ways. In the first, observations were made daily for three 15-min periods (morning, midday, and afternoon) throughout the duration of the experiment. In a second approach, crab activity was automatically recorded on video cassette for 40 s intervals, every half hour, 24 h a day. Using this latter method, up to eight animals could be monitored simultaneously.

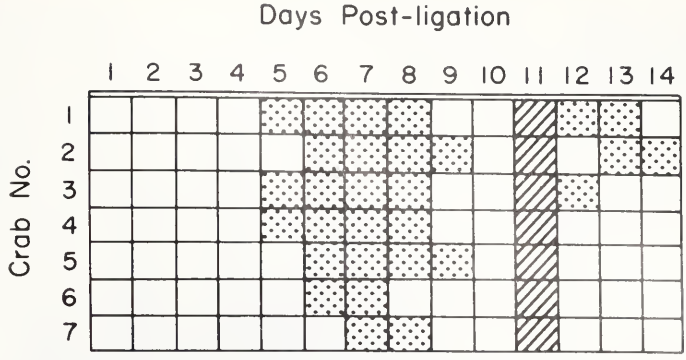

FIGURE 1. Occurrence of "spontaneous display behavior" (stippled squares) following bilateral eyestalk ligation of adult *Callinectes sapidus* males. All animals were observed for three 15 min periods daily. No data were collected on day 11.

Pheromone receptivity of males was tested by introducing a 300 μl sample of pubertal-female urine into the inlet flow of each aquarium and observing the male's behavior for two minutes. In all tests the urine samples were derived from pooled batches which, in our bioassay system, reliably trigger display behavior in intact, receptive males within two minutes.

For histological processing of the androgenic glands, the subterminal regions of the deferent ducts were dissected out, fixed with 2.5% glutaraldehyde in isosmotic *Callinectes* saline (Perkins and Wright, 1969) and refrigerated overnight. This was followed by treatment in Carnoy's fluid for 90 min at 4°C then dehydration and imbedding in paraffin. Tissue cross-sections (10 μm thick) were subsequently subjected to standard hematoxylin and eosin staining.

RESULTS AND DISCUSSION

A pilot study to examine the effects of eyestalk ligation on pheromone receptivity was performed using four non-receptive adult males. In these trials a pheromone stimulus was presented to each animal, daily, for several days following bilateral eyestalk ligation. The results were surprising. Exposure to the pheromone during the first four days post-ligation did not trigger courtship behavior; however, on days five and six, three of the males began to exhibit courtship display activity *in the absence of a pheromone stimulus.* This behavior was, in all respects, identical to the stereotyped display activity that is normally triggered by the pheromone of pubertal females. Carrying behavior, which normally follows courtship display, was also initiated by these spontaneously active animals when other males were placed in their tanks. Bouts of the "spontaneous display behavior" (SDB), lasting up to several hours, occurred for approximately three days; thereafter the activity appeared to decline. Because of this spontaneous behavior, the results of tests to evaluate pheromone receptivity in these animals were inconclusive.

To explore this phenomenon further, an experiment was conducted using 30 intermolt males of mature size (carapace short-width = 113.3 mm, SEM ± 0.8), 15 of which were randomly selected for bilateral eyestalk ligations; the remaining 15 served as controls. All animals were observed three times daily over a 14 day period to monitor the occurrence of SDB. Of the 10 males surviving ligation, 7 exhibited SDB, whereas no such activity was observed in the 15 control animals (Fig. 1). The sponta-

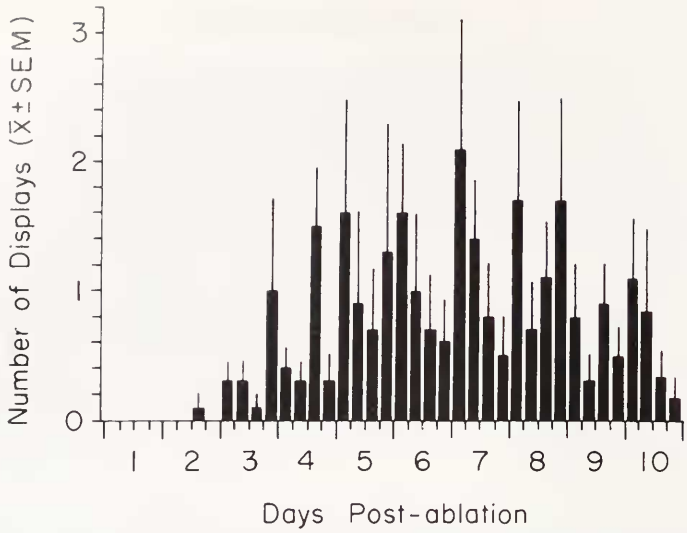

FIGURE 2. Frequency profile of "spontaneous display behavior" for 12 adult *Callinectes sapidus* males following bilateral eyestalk ablation. Behavior was sampled for 40 s intervals every half hour over 10 days. Shown are the mean number of intervals in which display activity occurred during each six hours post-ablation.

neous behavior was not seen until the fifth day after ligation, reaching a peak on day seven before subsiding.

The profile of this behavior was more precisely documented in a group of 12 adult, intermolt males (carapace short-width = 120.4 mm, SEM ± 1.2) in which the eyestalks were ablated. In this experiment, activity was automatically recorded on video cassette. The mean number of 40 s intervals in which SDB was observed during each 6 hours commencing on the day following ablation are shown in Figure 2. Again the profile reveals a delayed onset of the SDB with a maximum frequency between day five through eight, followed by a decline.

These results suggested that the eyestalks are sites for the production and/or release of a hormonal factor that modulates the activity of CNS pathways controlling courtship display behavior; *e.g.,* by regulating central pattern-generator circuits that coordinate the display. However, since the ligation/ablation procedure also removes a substantial portion of the crab's nervous system (*i.e.,* ganglia located in the eyestalks), an alternative hypothesis is that the procedure eliminates "inhibitory neural inputs" from these ganglia to courtship display "centers" located elsewhere in the CNS.

To distinguish between these alternatives, the optic tracts, which link the eyestalk ganglia with the brain (supraesophageal ganglion), were bilaterally transected in a group of 19 adult, intermolt males (carapace short-width = 117.8 mm, SEM ± 2.5). A second group of five adult, intermolt males (carapace short-width = 118 mm, SEM ± 4.7) received eyestalk ligations. The behavior of all animals was monitored three times daily over a ten day period. Dissection revealed that the transections were complete in 17 of the 19 animals subjected to the procedure, and in virtually all cases, hemolymph circulation to the eyestalks was not compromised as judged by the lack of necrosis in the eyestalk tissues 10 days after the operation. Of the 17 "transected" animals, only a single crab exhibited SDB. In contrast, four of the five "ligated" males

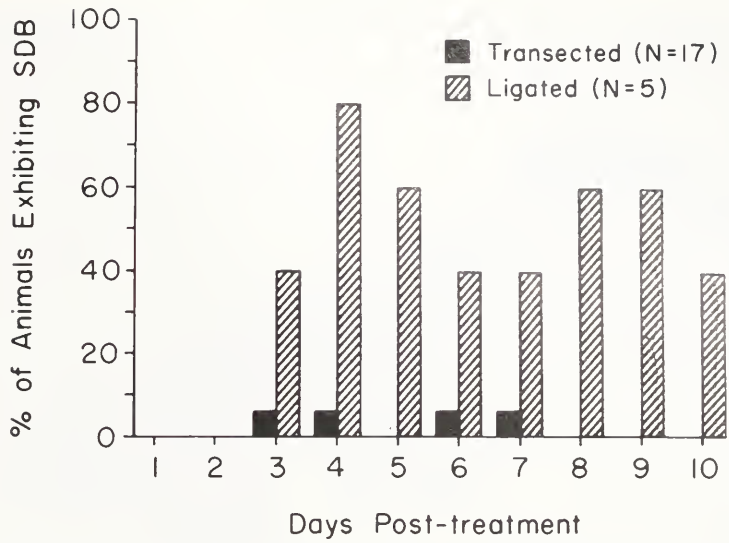

FIGURE 3. Occurrence of "spontaneous display behavior" (SDB) in adult *Callinectes sapidus* males following either bilateral eyestalk ligation (ligated) or optic tract transection (transected).

were induced to display (Fig. 3). The difference in the frequency of occurrence of SDB between experimental groups is significant ($P = 0.003$, Fisher exact test). These findings therefore demonstrate that the loss of any inhibitory neural inputs from the eyestalk ganglia is not a factor in the induction of SDB, and support the concept of an eyestalk hormone which acts to modulate this behavior. The latter view is a particularly tenable hypothesis considering that the transection procedure leaves only the integument and hemolymph linking the eyestalks to the animal. The operation therefore approximates (although it does not replace) the implantation of eyestalk tissue as a means of demonstrating the existence of a hemolymph-borne factor.

That the androgenic glands may be involved as possible intermediates in the induction of SDB is suggested by the hypertrophy they undergo following eyestalk ligation. The massive increase in size of the androgenic tissue depicted in Figure 4 is representative of what is consistently observed in adult, intermolt males subjected to the ligation procedure, and is quite apparent under the dissecting microscope as soon as day five following ligation. At least part of the increase in size can be attributed to hypertrophy of individual cells. Control animal tissue was characterized by cords of compact, linearly arranged cells; whereas in eyestalk-ligated males, the cytoplasm was typically more basophilic and greatly increased in volume. Under the light microscope, the appearance of the androgenic tissue in males actively exhibiting SDB was indistinguishable from that taken from animals up to 14 days post-ligation, a time at which the frequency of SDB activity has subsided. Hypertrophy was not observed in males in which the optic tracts were transected.

To assess the effects of eyestalk ligation on pheromone receptivity, 18 "ligated" males exhibiting SDB were screened for their response to pheromone-containing urine. The urine was presented during a period of quiescence between bouts of SDB. Only one of the eyestalk-ligated males, however, exhibited display behavior with urine presentation, and the response latency suggested that it may have been a bout of SDB. To explore this deficit in receptivity further, a group of seven highly receptive,

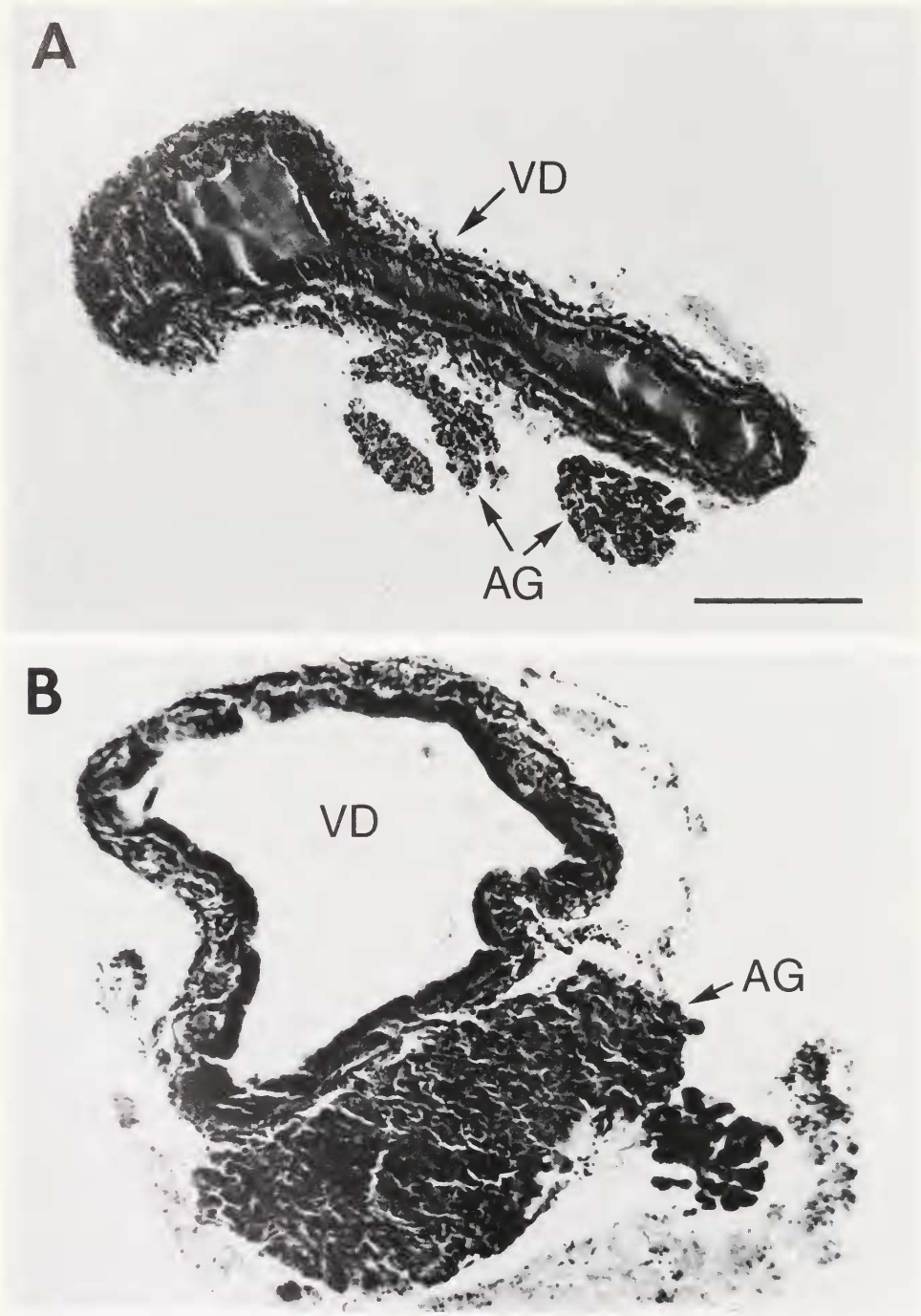

FIGURE 4. Cross-sections of the posterior vas deferens (VD) with associated androgenic gland (AG) from a control (A) and experimental male (B) 14 days following bilateral eyestalk ligation. Scale bar equals 200 microns.

intact males, which repeatedly exhibited vigorous display activity with urine presentations, were tested 24 h after either bilateral eyestalk ligation (two animals) or optic tract transection (five animals). In these trials, the pheromone stimulus did not induce any of the animals to display.

These results suggest, first, that disrupting the neural connections between the eyestalks and the supraesophageal ganglion blocks chemosensory activation of courtship display "centers" in the CNS; thus implying that neural pathways within the eyestalk ganglia are, indeed, important for processing or transmitting pheromone stimulus information. Two lines of reasoning support this view: (1) males which reliably respond to a pheromone stimulus immediately lose their receptivity if the neural connections to the eyestalks are severed; and (2) since the motor program for display behavior remains intact following eyestalk ligation/ablation as revealed by SDB activity, the lack of response to a pheromone stimulus in eyestalk-lesioned males cannot be attributed to the animal's inability to perform the display, implying that chemosensory activation of the program is in some way blocked. Interestingly, in several crustaceans bilateral eyestalk lesions have also been shown to profoundly attenuate food-search behavior elicited by chemical stimulation of the antennules (Maynard and Sallee, 1970; Hazlett, 1971). A second point to be made from these data is that the SDB observed in ligated males is not attributable to hyperexcitability of pheromone receptor cells in the antennules (although such hyperexcitability may in fact also occur), but rather involves changes in the excitability of CNS pathways mediating display behavior. This is further supported by the fact that in nine eyestalk-ligated males in which the antennules were also bilaterally ablated, SDB was nevertheless induced.

Considering the data together we propose the following relationships as a working hypothesis (Fig. 5). In receptive males, stimulation of antennular pheromone receptors normally activates CNS "centers" controlling courtship display; and in the pathway linking the antennular receptors with these "centers," neural connections within the eyestalk ganglia appear to be important components. We propose that the excitability of the display "centers," and consequently the threshold for activation by a pheromone stimulus, is modulated by an eyestalk hormone; possibly a neurosecretory product released into the hemolymph via the sinus gland. Exactly how this modulation is effected is not known. The action could be direct [*e.g.,* via the action of neuro-depressing hormone (Arechiga *et al.,* 1977, 1979)], and/or entail one or more intermediates. One possibility of the latter is that a hormone(s) from the androgenic glands is involved, as is suggested by the hypertrophy these glands undergo following eyestalk ligation. Indeed, a positive correlation between androgenic gland size and seasonal changes in the testes (Meusy, 1963; Payen, 1973), vas deferens (Adiyodi, 1985), and external male morphology (Carpenter and DeRoos, 1970; Thampy and John, 1973; Dudley and Jegla, 1978) has been noted in other crustaceans. Thus, if an androgenic hormone acts to turn on or increase the excitability of CNS courtship-display "centers," the eyestalk hormone may, in fact, be acting indirectly on these "centers" by modulating androgenic gland activity. Alternatively, the action of the eyestalk hormone may be mediated via other pathways; for example, by regulating the production or activity of a gonad stimulating hormone having CNS actions (Adiyodi and Adiyodi, 1970; Eastman-Reks and Fingerman, 1984; Kulkarni *et al.,* 1984).

The temporal profile of the SDB activity is intriguing and will quite likely provide an important clue towards understanding the mechanisms underlying this phenomenon. For example, the latency to onset of SDB may reflect the time required to appropriately raise the titer of a circulating androgenic hormone and/or reflect the time course of metabolic processes giving rise to the hyperexcitability in the neural elements affected by the hormone. The decline in SDB frequency by day 10 post-ligation

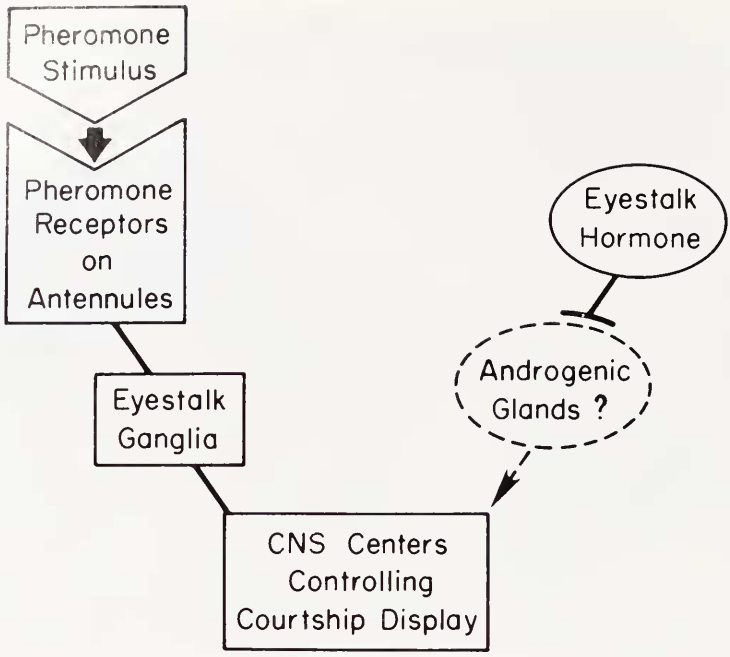

FIGURE 5. Proposed model for activation and modulation of CNS "centers" controlling courtship display behavior. See text for discussion.

(Figs. 1, 2) may result from desensitizing mechanisms within the CNS or from compensatory metabolic processes which reduce the circulating titer of hormone. If the androgenic glands are indeed involved in the induction of SDB, the fact that their hypertrophy (and presumably hypersecretion) is evident well past the peak in SDB activity would suggest that mechanisms which compensate for a high titer of circulating androgenic factor are responsible for the reduction in SDB by day 10. Certainly, deciphering the exact mechanisms underlying SDB should provide important insight towards understanding processes regulating the sensory activation of behavior.

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